PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/00, 5/08, C07K 13/00 A61K 37/36, 31/505, 35/14	A1	(11) International Publication Number: WO 92/01039 (43) International Publication Date: 23 January 1992 (23.01.92)

(21) International Application Number: PCT/US91/04885

(22) International Filing Date: 10 July 1991 (10.07.91)

(30) Priority data:
552,746 13 July 1990 (13.07.90) US

(71) Applicants: DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US). DUKE UNIVERSITY [US/US]; 07 Allen Building, Durham, NC 27706 (US).

(72) Inventors: SAGER, Ruth ; 30 Codman Road, Brookline, MA 02146 (US). TRASK, Douglas, K. ; Apt. 311A, 1550 Brickell Avenue, Miami, FL 33129 (US). LE, Phong ; 3315 Coachmans Way, Durham, NC 27705 (US).

(74) Agent: FRASER, Janis, K.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: PROMOTION OF MATURATION OF HEMATOPOIETIC PROGENITOR CELLS

(57) Abstract

A method for promoting maturation of a hematopoietic precursor cell of an animal, which method includes the step of contacting the cell with a maturation-promoting amount of GRO, a polypeptide growth factor.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CS	Czechoslovakia
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GN	Guinea
GR	Greece
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
PL	Poland
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

PROMOTION OF MATURATION OF HEMATOPOIETIC PROGENITOR CELLS

Background of the Invention

This work was supported by an Outstanding Investigator Grant, CA 39814, to Dr. Ruth Sager from the United States Government, which has rights in the invention. This invention relates to methods for promoting maturation of hematopoietic precursor cells.

GRO is a polypeptide growth factor encoded by a gene termed *gro* (Anisowicz et al., Proc. Natl. Acad. Sci. USA 84:7188-7192, 1987; Anisowicz et al. Proc. Natl. Acad. Sci. USA 85:9645-9649, 1988). The amino acid sequence of mature human GRO (i.e., the sequence deduced by Anisowicz et al., 1987, from the *gro* cDNA sequence, but minus the 34-amino acyl residue leader peptide) is identical to a 73-amino acyl residue-long protein present in the conditioned medium of a human malignant melanoma cell line, and which has been termed melanoma growth stimulation activity, or "MGSA" (Richmond et al., Cancer Research 43:2106-2112, 1983; Richmond et al., EMBO J. 7:2025-2033, 1988). Likewise, a protein secreted by activated neutrophils (neutrophil-activating peptide-3 or NAP-3) has an amino-terminal sequence identical to that of GRO/MGSA, at least up to the 31st residue of each (Schroder et al., J. Exp. Med. 171:1091-1100, 1990). GRO/MGSA acts as a growth factor for melanoma cells, lung carcinoma cells, nevus cells, and some immortalized fibroblast cell lines (Richmond and Thomas, J. Cell Biol. 107:40A #203, 1988).

The *gro* genes exhibit DNA sequence similarities to a family of genes encoding secretory proteins associated with the inflammatory response. The expression of each gene of this family of genes, including *gro*, is rapidly induced in susceptible cells by such agents as phorbol esters, interleukin-1, and tumor necrosis factor (TNF), as well as other cytokines and growth factors (Anisowicz et al., 1987; Anisowicz et al., 1988). Members of this family encode proteins which exhibit a high degree of sequence homology, including four cysteine residues present in analogous positions; such proteins include platelet factor 4 (Deuel et al., Proc. Natl. Acad. Sci. USA 74:2256, 1977); platelet basic protein and its cleavage products: β-thromboglobulin (Begg et al., Biochemistry 17:1739, 1978) and connective tissue activity peptide III (CTAP III) (Castor et al., Proc. Natl. Acad. Sci USA 80:765, 1983); interferon inducible protein 10 (IP10) (Luster et al., Nature 315:672, 1985); macrophage inflammatory protein-2 (MIP-2) (Wolpe et al., Proc. Natl. Sci. USA 86:612, 1989); and neutrophil activating peptide-1/interleukin-8 (NAP-1/IL-8) (Walz et al., Biochem. Biophys. Res. Commun. 149:755, 1987) [also known as NAF (Walz et al.; Lindley et al., Proc. Natl. Acad. Sci. USA 85:9199, 1988), MDNCF (Yoshimura et al., Proc. Natl. Acad. Sci. USA 84:9233, 1987), MONAP (Schroder et al., J. Immunol. 139:3474, 1987), and GCP (Van Damme et al., J. Exp. Med. 167:1364, 1988)]. This latter compound is a neutrophil-specific chemotactic factor (Walz et al.; Lindley et al.; Yoshimura et al.; Schroder et al.; and Van Damme et al.) and cellular activator (Wolpe et al.; Lindley et al.), as well as an inhibitor (at low concentrations) (Gimbrone et

al., Science 246:1601, 1989) and activator (at high concentrations) (Carveth et al., Biochem. Biophys. Res. Commun. 162:387, 1989) of neutrophil adhesion to endothelial cells.

Based on what is said to be largely indirect evidence, Anisowicz et al. (1988) suggest that the *gro* gene may play a role "in a variety of important cellular functions: as a putative [positive] early response gene in cell growth, as a mediator of the IL-1-induced inflammatory response in fibroblasts, and as a negative regulatory factor in epithelial cells."

Summary of the Invention

In general, the invention features a method for promoting maturation of a hematopoietic precursor cell (preferably a CFU-GEMM cell or a CFU-GM cell) of an animal (e.g., a human), which method includes the step of contacting the cell with a maturation-promoting amount of GRO. In preferred embodiments, the cell is first removed from the animal (e.g., by withdrawing bone marrow from a bone of the animal), and is contacted with GRO *in vitro*; the cell and/or its descendants are then preferably reinserted into the animal, or alternatively into a second animal (most preferably a human).

Also featured is a method for promoting the maturation of a hematopoietic precursor cell (preferably a CFU-GEMM cell or a CFU-GM cell) within an animal (e.g., a human) by treating the animal with a maturation-promoting amount of GRO. The form of GRO utilized in any method of the invention is preferably a recombinant GRO (i.e., produced by expression of a recombinant DNA molecule encoding GRO), and may, for example, have the amino acid

sequence of a mature human GRO, or may be an analog or fragment of a naturally-occurring GRO.

Another method of promoting the maturation of a hematopoietic precursor cell within an animal is to introduce into the animal one or more cells capable of excreting GRO (i.e., secreting or otherwise causing GRO to be exported out of the cell); alternatively, a gene encoding and capable of expressing GRO can be introduced into one or more cells of the animal, to form a cell (herein termed a "transgenic cell") capable of excreting GRO within the animal at a level sufficient to promote maturation of the hematopoietic percursor cell.

The term "hematopoietic cells" herein refers to fully differentiated myeloid cells such as erythrocytes or red blood cells, megakaryocytes, monocytes, granulocytes, and eosinophils, as well as fully differentiated lymphoid cells such as B lymphocytes and T lymphocytes; it also encompasses the various hematopoietic precursor cells from which these differentiated cells develop, such as BFU-E (burst-forming units-erythroid), CFU-E (colony forming unit-erythroid), CFU-Meg (colony forming unit-megakaryocyte), CFU-GM (colony forming unit-granulocyte-monocyte), CFU-Eo (colony forming unit-eosinophil), and CFU-GEMM (colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte). The interrelationships among these hematopoietic cells and their positions along the various paths of differentiation are illustrated in Fig.1. "Maturation" of a hematopoietic precursor cell is used herein to mean the generation of descendents of such precursor cell which are either identical to or more differentiated than such precursor cell, or a mixture of both. For example, a CFU-GEMM would be

induced by the method of the invention to generate multiple cells, some of which are CFU-GEMMs and others of which are further along the paths of differentiation, such as CFU-GM, CFU-Eo, and megakaryocytes, or are fully differentiated, end-stage cells such as monocytes/ macrophages, platelets, or granulocytes (e.g., neutrophils, basophils, or eosinophils). A maturation-promoting amount of GRO is that amount of protein which is sufficient to cause maturation of a significant number of hematopoietic precursor cells present in a bone marrow or taken from a bone marrow. For example, out of a population of approximately 10^6 light-density bone marrow cells (i.e., bone marrow cells which do not pellet in the Ficoll-Hypaque method described below) plated on a semi-solid substrate, at least one hematopoietic precursor cell would be induced by this amount of GRO to proliferate until the cell had formed a colony (a group of cells all of which are descended from a single cell) of at least 8 cells (after 7 days incubation at 37°C) or at least 40 cells (after 14 days incubation at 37°C), which cells are identical to or more differentiated than the precursor cell from which they were derived. For *in vivo* treatment with GRO, a maturation-promoting amount of GRO would be an amount capable of increasing the number of hematopoietic cells in the treated patient.

The ability of a given form or amount of GRO to promote maturation of cells can be measured by any standard procedure. For example, this biological activity can be measured *in vitro* by measuring the colony-promoting activity of GRO on cells taken from a bone marrow and grown on a semi-solid substrate as described below.

By GRO is meant not only the MGSA protein of Richmond et al. (1983, 1988) and the *gro*-encoded protein of Anisowicz et al. (1987, 1988), but also any comparably active GRO endogenous to any animal species (particularly mammals or other vertebrate species). Three different human *gro* cDNAs have been cloned. Anisowicz et al. (1987) identified the first (now termed *gro* α) from a human bladder carcinoma cell line (T24) cDNA library. An adherent monocyte cDNA library probed with *gro* α cDNA yielded an 880 bp partial cDNA clone, the sequence of which differed somewhat from that of *gro* α cDNA; this partial cDNA was used to probe a second cDNA library, producing positively-hybridizing clones representing *gro* α cDNA and two variants termed *gro* β and *gro* γ. Partial sequence analysis of genomic clones obtained by probing a human genomic DNA library with *gro* α cDNA confirmed that the three forms are derived from related but different genes, all three of which appear to map to the same region of chromosome 4q. The nucleotide sequences and predicted translation sequences of the three cDNAs are compared in Fig. 2.

Genes or cDNAs encoding such alternative naturally-occurring GROs may be identified and cloned using human or other *gro* cDNA as a hybridization probe, in a manner similar to that employed by Anisowicz et al. (1987). The cDNA sequences and corresponding amino acid sequences for human GRO α and for Chinese hamster GRO, as published by Anisowicz et al. (1987), are set forth in Fig. 3. The term GRO also encompasses any analog or fragment of any naturally-occurring GRO, which analog or fragment is stable in solution and exhibits a maturation-promoting biological activity

comparable to that of the naturally-occurring GRO/MGSA of which it is an analog or fragment. It is critical only that the maturation promoting portion of GRO/MGSA be provided. The critical portion of GRO/MGSA can be determined by any of a number of standard techniques. For example, the cDNA or cloned gene encoding GRO may be modified by standard *in vitro* mutagenesis techniques to cause expression of a GRO analog with amino acid substitutions, additions, and/or deletions of one or more amino acids at one or more locations. The amino acid substitutions may be either conservative or non-conservative, and may be designed, for example, to remove proteolytically sensitive sites from the GRO protein. [By conservative is meant that the substituted amino acyl residue is chemically similar (e.g., acidic, basic, hydrophobic, aromatic) to the residue for which it is substituted: for example, substitution of a valine for a leucine.] Examples of GRO proteins with potentially useful additions would include a GRO with a short peptide added to either terminus, such as a leader peptide to facilitate secretion of the protein out of the cell, or a peptide added by means of genetic engineering to provide an antigenic site to permit immunoaffinity-based purification of the protein product; and chimeric GRO proteins covalently bound to polypeptide ligands capable of binding to particular receptors. Forms of GRO with internal amino acid additions which do not destroy the maturation promoting activity are also within the definition of GRO. Once generated, any such analogs can then be tested for the desired biological activity, i.e. maturation promoting activity. In this way, the maturation promoting

portion of GRO can be specifically determined, and those amino acyl residues not critical to that function removed or replaced with other residues. Alternatively, the naturally-occurring or recombinant protein may be digested with a variety of proteases, for example, trypsin, to provide fragments which can then be tested for maturation promoting activity. Those fragments or analogs which have the maturation promoting portion of naturally-occurring GRO can be readily determined using simple *in vitro* techniques.

Also included by the term GRO is a protein or polypeptide having an amino acid sequence of between 70 and 100 amino acyl residues with either (a) a contiguous 20-residue segment thereof having at least 80% sequence homology with a portion of a naturally-occurring mature GRO (i.e., when the 20-residue segment is lined up with such portion of a naturally-occurring mature GRO, at least 80% of the residues of the former will be identical to the corresponding residues of the latter), or (b) a contiguous 10-residue segment thereof having at least 90% sequence homology with a portion of a naturally-occurring mature GRO. If the GRO polypeptide is of lesser size than 70 amino acids (for example 20 to 30 amino acids), such polypeptide will have at least 80% sequence homology with some portion of the naturally-occurring GRO. GRO can be produced by any standard technique, including by extraction from animal tissues or cells which naturally produce the protein or can be induced to do so, by chemical synthesis, and by recombinant DNA technology. As discussed above, the DNA encoding the desired GRO can be modified by standard techniques to encode a GRO having a different amino

acid sequence from that described by Richmond et al., 1988, and Anisowicz et al., 1987, and may be expressed in any desired cell type. It is not necessary that GRO be produced in a glycosylated state, since the naturally-occurring protein is not glycosylated.

Applicants have surprisingly discovered that GRO is useful for promoting maturation of certain hematopoietic precursor cells. Previously, factors which were useful for promoting hematopoietic precursor cell maturation included colony-stimulating factors (CSFs) such as multi-CSF (also termed interleukin-3 or IL-3), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF). All of these are glycoproteins of molecular weight 14-45 kD that are synthesized by multiple cell types including endothelial cells, fibroblasts, macrophages, and lymphocytes. In contrast, GRO is not a glycosylated protein and has a molecular weight of only 7 kD. Further, its amino acid sequence is very different from the respective sequences of the known CSFs. Other factors which have been found to work in synergy with the CSFs include IL-1 and IL-6, neither of which has an amino acid sequence similar to that of GRO.

The method of the invention provides a way to boost a patient's level of fully differentiated hematopoietic cells (such as granulocytes and macrophages) by inducing the proliferation and maturation of hematopoietic precursor cells. Therapy with GRO can be accomplished either *in vivo* or *ex vivo*, and can utilize the patient's own bone marrow cells or cells provided by a donor. GRO may

be used alone or in combination with other growth factors/cytokines such as the interleukins (particularly IL-1, IL-3, IL-6, and IL-8), the colony stimulating factors (e.g., GM-CSF, G-CSF, and M-CSF), and erythropoietin, in order to achieve optimal clinical results.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings are first briefly described.

Fig. 1 is a diagram illustrating the interrelated paths of differentiation of various hematopoietic cells. (taken from a 1990 illustration produced by Schering Plough Corporation and Sandoz Pharmaceuticals Corporation).

Fig. 2 is a comparative representation of the cDNA sequences of human *gro* α, *gro* β, and *gro* γ: (A) the coding sequences, with corresponding amino acid sequences; (B) the 5' untranslated sequences; and (C) the 3' untranslated sequences, with a conserved region shown boxed.

Fig. 3 is a representation of the nucleotide sequences and corresponding amino acid sequences for human GRO α and Chinese hamster GRO: (A) 5' untranslated and protein coding regions. (B) 3' untranslated regions. (C) regions of significant homology between the human and the Chinese hamster *gro* cDNA 3' untranslated regions. (Taken from Figs. 4 and 5 of Anisowicz et al., 1987.)

Fig. 4 is a graph showing the chemotactic activity of recombinant GRO (rgro) and a known chemoattractant,

f-Met-Leu-Phe (fmlp), for polymorphonuclear leukocytes (PMNs).

Preparation of GRO

As stated above, GRO may be prepared by any standard method, including but not limited to those utilizing recombinant DNA techniques. Two independent preparative methods are described below in Examples 1 and 2, but alternative methods will be apparent to those of ordinary skill in the art of protein production.

Example 1

COS-1 cells (ATCC No.CRL1650) were transiently transfected with a pXM expression vector (available from Genetics Institute, Cambridge, MA) containing human *gro* cDNA (Anisowicz et al., 1987). Cells were maintained for one day in Alpha medium (Gibco) containing 10% fetal calf serum (FCS), and then were washed with serum-free medium and maintained for two days in Alpha salts plus 100 U/ml penicillin and 100 μg/ml streptomycin. Culture medium was harvested and subjected to low-speed centrifugation (500 x g for 5 min) to pellet cellular debris. The culture supernatant was dialyzed against 10mM sodium phosphate, pH 6.5, and applied directly to a cation exchange column (CM-Sephadex, Pharmacia, Uppsala, Sweden). The bound proteins were eluted with a linear salt gradient of 0 to 0.7 M NaCl in 10 mM sodium phosphate, pH 6.5. GRO-containing fractions were determined by analyzing aliquots of each fraction on 18% SDS-polyacrylamide gels, with a distinct silver-staining band at 6kD indicative of the presence of mature recombinant GRO. The identity of the GRO-containing band was confirmed by positive cross-reactivity with an antibody prepared against a 10-amino acyl residue carboxy-terminal

fragment of GRO. GRO-containing fractions were pooled and the protein was concentrated by differential filtration through a membrane with a molecular weight cut-off of 3,000 daltons.

Example 2

An analog of mature human GRO [differing from naturally-occurring mature human GRO α by an amino-terminal octapeptide tag: AspTyrLysAspAspAspAspLys (Hopp et al., Biotechnology 6:1204-1210, 1988)] was produced in yeast by recombinant DNA techniques. A segment of *gro* cDNA encoding mature human GRO α was inserted into the yeast expression plasmid pαADH2 (Price et al., Gene 55:287-293, 1987) and expressed in *S. cerevisiae* strain XV218/(a/α-trp-1). The resulting GRO analog was purified in a one-step immunoaffinity procedure utilizing an antibody specific for the first four residues of the octapeptide tag, according to the method of Hopp et al.

Biological Assays for GRO

GRO (including fragments and analogs of a naturally-occurring GRO) may be assayed for biological activity by a method such as one described in Examples 3-5.

Example 3: Assay for Maturation Promoting Activity

The ability of GRO to promote maturation of hematopoietic precursor cells can be conveniently assayed *in vitro* by an assay such as the one herein described. Functional variations on this assay, and alternative assays, will be apparent to those of ordinary skill in the art.

Bone marrow from a normal human or other animal is harvested by standard sterile procedures, heparinized, and either frozen or used immediately. Light-density bone marrow cells are isolated by

density gradient centrifugation on Ficoll-Hypaque (LSM, Organia-Technica, Durham, NC) according to standard methods, and then washed and adjusted to 1 x 10^6 cell/ml in RPMI 1640 medium (Gibco) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 12.5% FCS, and 12.5% horse serum. The cells (1 x 10^5) are plated in duplicate on Lux 35-mm gridded culture dishes (Nunc, Inc., Naperville, IL) in 1.0 ml of the same culture medium containing methylcellulose (1500 centipoise) at a final concentration of 0.9% (w/v). The GRO preparation to be tested is added to a final concentration of, for example, 25, 50, or 100 ng/ml. The dishes are then placed in a 150 mm dish with water for humidification, and incubated in 5% CO_2 in air at 37°C. After 14 days of incubation, plates are scored for hematopoietic cell colonies (≥ 40 cells/ aggregate). Each colony generally contains a mixture of hematopoietic cell types, with the particular combination of types present in a given colony indicative of the identity of the hematopoietic precursor cell from which the colony descended. For example, a colony which contains only granulocytes, monocytes, and/or CFU-GM cells arises by the action of a maturation-promoting activity on a single CFU-GM cell and would be scored as a CFU-GM colony, while a second colony which contains those three cell types plus eosinophils, CFU-Eo, megakaryocytes, erythrocytes, CFU-Meg, CFU-E, BFU-E, and/or CFU-GEMM is descended from a more primitive precursor cell, a CFU-GEMM, and so would be scored as a CFU-GEMM colony. Methods of distinguishing one hematopoietic cell type from another are well known to those of ordinary skill in the field of hematology. For example, the cellular morphology of individual colonies may be evaluated by differential counts performed on cytocentrifuge

preparations of cells stained with the Diff-Quick modification (Sigma Chemical Co., St. Louis, MO) of the Wright's Giemsa technique.

As a negative control, an equivalent aliquot of buffer lacking GRO (or any cytokine) is added to similarly prepared bone marrow cells. Positive controls vary with the specific type of hematopoietic precursor cell being analyzed for colony formation. In the experiments set forth in Table I below, recombinant human GM-CSF at 5 U/ml (Genetics Institute, Cambridge, MA) was used as a positive control for induction of colony formation by CFU-GM cells, while a combination of 2 U/ml recombinant human erythropoietin (Amgen, Thousand Oaks, CA), 0.5 mM 2-mercaptoethanol, and conditioned culture medium from the human bladder carcinoma cell line 5637 (Welte et al., Proc. Natl. Acad. Sci. USA 82:1526, 1985) was used as a positive control for induction of colony formation by both CFU-GEMM and burst forming unit-erythroid (BFU-E) cells. The choice of other cytokines as positive controls for induction of formation of colonies by other types of hematopoietic cells is within the skills of those in the field to which the invention pertains.

The results of six separate experiments using the above-described *in vitro* assay to test the bioactivity of recombinant human GRO α are shown in Table I below. The number of colonies formed after incubation for 14 days in the presence of each concentration of GRO tested (100 ng/ml, 50 ng/ml and 25 ng/ml) is expressed as a percentage of the colonies formed after incubation for a similar period in the presence of the applicable positive control cytokine (as described above). Under these culture conditions, the positive controls generate approximately 100-250 CFU-GM colonies, 24-60 CFU-

GEMM colonies, and 40-200 BFU-E colonies per 10^6 bone marrow cells plated. Values for negative control plates are shown immediately below each corresponding GRO test result. The results show that recombinant human GRO is capable of stimulating generation of colonies of CFU-GM cells, CFU-GEMM cells, and possibly BFU-E cells in a bone marrow cell preparation, in some cases to an extent greater than that seen in the positive control.

TABLE I

COLONY FORMATION BY BONE MARROW CELLS TREATED WITH GRO

	Number of colonies/10^6 light-density bone marrow cells expressed as a % of (+)control		
	CFU-GM	CFU-GEMM	BFU-E
Experiment #1			
GRO (100 ng/ml)	87%	103%	70%
Buffer (1:15)	76	32	34
GRO (50 ng/ml)	98	64.5	64
Buffer (1:30)	67	26	27
GRO (25 ng/ml)	76	48.4	56
Buffer (1:60)	70	16	15
Experiment #2			
GRO (100 ng/ml)	69	82	82
Buffer (1:15)	35	35	64
GRO (50 ng/ml)	ND	ND	ND
Buffer (1:30)	18	41	9
GRO (25 ng/ml)	102	88	86
Buffer (1:60)	18	24	18
Experiment #3			
GRO (100 ng/ml)	117	116	99
Buffer (1:15)	69	37	84
GRO (50 ng/ml)	172	130	74
Buffer (1:30)	74	63	63
GRO (25 ng/ml)	109	137	93
Buffer (1:60)	87	87	28

TABLE I (continued)

COLONY FORMATION BY BONE MARROW CELLS TREATED WITH GRO

	Number of colonies/10^6 light-density bone marrow cells expressed as a % of (+)control		
	CFU-GM	CFU-GEMM	BFU-E
Experiment #4			
GRO (100 ng/ml)	115%	125%	65%
Buffer (1:15)	38	25	10
GRO (50 ng/ml)	91	108	45
Buffer (1:30)	35	17	15
GRO (25 ng/ml)	62	75	15
Buffer (1:60)	29	33	10
Experiment #5			
GRO (100 ng/ml)	72	68	9
Buffer (1:15)	58	19	5
GRO (50 ng/ml)	66	50	14
Buffer (1:30)	38	19	2
GRO (25 ng/ml)	57	69	5
Buffer (1:60)	35	31	2
Experiment #6			
GRO (100 ng/ml)	75	7	40
Buffer (1:15)	14	0	5
GRO (50 ng/ml)	83	4	15
Buffer (1:30)	18	1	0
GRO (25 ng/ml)	98	12	25
Buffer (1:60)	7	1	0

Example 4: Assay Following Depletion of Accessory Cells

In order to determine whether or not GRO stimulates proliferation by direct action on the proliferating cells, or by inducing production of an undetermined cytokine by other "accessory" cells present in the bone marrow preparation, an accessory cell depletion experiment was carried out. Light-density bone marrow cells were prepared as described above and depleted of monocytes by exposing the preparation to a plastic Petri dish for 3 hours to permit adherence of monocytes. Non-adherent cells were then removed from the dish, washed, and subjected to further analysis. Depletion of NK (natural killer) and T lymphocytes was accomplished by E rosetting by the method of Elliott and Pross (Methods in Enzymology 108:49-64, 1984). The results of treating such depleted bone marrow cell populations with recombinant GRO, shown in Table II, indicate that GRO retains most or all of its ability to stimulate maturation of CFU-GM cells even in the absence of monocytes and/or NK and T cells, suggesting that GRO may act directly on the proliferating CFU-GM cells. In contrast, the ability of GRO to stimulate formation of colonies by CFU-GEMM appears to be decreased in the absence of such accessory cells. This result suggests that GRO affects CFU-GEMM cells at least in part by an indirect route that involves other cells, perhaps by inducing accessory cells to produce a different growth-stimulating cytokine.

TABLE II

COLONY FORMATION BY ACCESSORY CELL-DEPLETED BONE MARROW CELLS TREATED WITH GRO

	Number of colonies/10^6 (pre-depletion) bone marrow cells, expressed as a % of (+)control		
	CFU-GM	CFU-GEMM	BFU-E
Experiment #7			
Monocyte-depleted			
GRO (100 ng/ml)	121%	18%	16%
GRO (50 ng/ml)	80	11	16
GRO (25 ng/ml)	78	46	8
(-)control (buffer)	26	7	0
NK+T cell-depleted			
GRO (100 ng/ml)	93	79	20
GRO (50 ng/ml)	93	114	17
GRO (25 ng/ml)	101	129	34
(-)control (buffer)	31	7	2
Experiment #8			
Monocyte-depleted			
GRO (100 ng/ml)	128	41	40
GRO (50 ng/ml)	124	72	27
GRO (25 ng/ml)	131	45	19
(-)control (buffer)	56	24	6
NK+T cell-depleted			
GRO (100 ng/ml)	115	71	38
GRO (50 ng/ml)	142	71	23
GRO (25 ng/ml)	104	150	54
(-)control (buffer)	51	21	0

TABLE II (continued)

COLONY FORMATION BY ACCESSORY CELL-DEPLETED BONE MARROW CELLS TREATED WITH GRO

	Number of colonies/10^6 (pre-depletion) bone marrow cells, expressed as a % of (+)control		
	CFU-GM	CFU-GEMM	BFU-E
Experiment #9			
NK+T cell-depleted			
GRO (100 ng/ml)	112%	400%	13%
GRO (50 ng/ml)	75	300	17
GRO (25 ng/ml)	46	400	20
(-)control (buffer)	31	0	0
Monocyte+NK+T cell-depleted			
GRO (100 ng/ml)	82	100	5
GRO (50 ng/ml)	71	250	5
GRO (25 ng/ml)	87	100	5
(-)control (buffer)	23	0	5
Experiment #10			
NK+T cell-depleted			
GRO (100 ng/ml)	89	0	0
GRO (50 ng/ml)	83	0	0
GRO (25 ng/ml)	58	0	0
(-)control (buffer)	20	0	0
Monocyte+NK+T cell-depleted			
GRO (100 ng/ml)	58	11	3
GRO (50 ng/ml)	26	11	3
GRO (25 ng/ml)	26	0	0
(-)control (buffer)	9	0	0

Example 5: Chemotaxis Assay

Certain members of the gene family to which the *gro* genes are related encode proteins which are chemotactic for neutrophils. In order to determine whether or not recombinant GRO is also chemotactic for such cells, the following assay was carried out:

Human peripheral venous blood taken from normal volunteers was subjected to dextran sedimentation (Pharmacia, Uppsala, Sweden) followed by centrifugation on Ficoll-Hypaque (Lympho-prep, Organon Technica, Durham, NC) as described by Williams et al. (Proc. Natl. Acad. Sci. USA 74:1204, 1977). Pellets containing polymorphonuclear leukocytes (PMNs, a type of neutrophil) were subjected to hypotonic lysis (x2); they contained greater than 95% PMNs as judged by microscopic examination of Wright's stained specimens. The buffy coat containing mononuclear cells was washed twice with Hank's balanced salt solution (Gibco, Grand Island, NY) containing 0.01 M HEPES, pH 7.0; 4.3 mM $NaHCO_3$ (HBSS) and 2% bovine serum albumin (HBSS-BSA). Mononuclear cell preparations contained 25-35% monocytes as determined by nonspecific esterase staining. Chemotaxis was quantified using 48-well microchambers (Neuroprobe, Inc., Cabin John, MD) (Harvath et al., J. Immunol. Methods 37:39, 1980; Falk et al., J. Immunol. Methods 33:239, 1980). PMNs (0.05 ml, 1 x 10^6/ml) suspended in HBSS, pH 7.2, were placed in the upper wells of the microchamber. HBSS alone or HBSS containing GRO or another chemotactic stimulant (0.03 ml) was placed in each of the lower chambers and was separated from the cells by a 3.0μm pore diameter polyvinyl pyrrolidine (PVP)-free polycarbonate filter (25 mm x 80 mm). Following incubation at 37°C in humidified

air for 60 min, the non-migrated cells were removed from the top of the filter, as counted and the migrated cells were stained with a leukocyte-specific stain (Leuko Stat, Fisher Scientific, Orangeberg, NY). PMN chemotaxis was quantified as the average number of cells/field which had migrated completely through the filter, as counted in ten oil immersion (x 1000) fields. Assays were performed in triplicate and expressed as the mean cells/field ± S.D. As shown in Fig. 4, recombinant human GRO increased the number of migrated PMNs approximately 2.8-fold when compared to HBSS alone. GRO induced significant migration of PMNs in concentrations ranging from 0.05 nM to 5.0 nM. Maximal neutrophil chemotactic activity was obtained at a GRO concentration of 0.7 ± 0.2 nM, with an effective concentration which produced 50% of maximal migration (EC_{50}) of 0.07 ± 0.05 nM. The total number of cells migrating in response to GRO ranged from 53% to 83% of the number of cells responding to 100 nM f-Met-Leu-Phe, a known chemoattractant. Monocyte chemotaxis was similarly quantified in the 48-well microchambers except that cells were suspended to 1.5×10^6 monocytes/ml in HBSS-BSA, pH 7.0; 5.0 μm PVP-coated polycarbonate filters were used; and incubations were allowed to proceed for 90 min in 37°C humidified air. Unlike human PMNs, human monocytes did not respond chemotactically to concentrations of GRO ranging from 0.01 nM to 10 nM (data not shown).

These results, analogous to those produced by NAP-3 and NAP-1/IL-8, suggest a role for GRO in acute inflammation.

Use

The maturation promoting ability of GRO is useful for treatment of a variety of diseases and conditions. For example, it can be used to promote regeneration of hematopoietic cells between cycles of myelotoxic chemotherapy, permitting use of increased doses of chemotherapeutic agents. The protein may also be used during or after autologous, allogeneic, or even xenogeneic bone marrow transplantation to promote accelerated engraftment. In addition, a number of genetic diseases characterized by neutropenia and thrombocytopenia can be treated by promoting the maturation of appropriate hematopoietic precursor cells. Continuous low-dose therapy will result in an increase in the patient's level of durable neutrophils and platelets. Azidothymidine (AZT) treatment of acquired immunodeficiency syndrome (AIDS) patients can induce severe neutropenia and anemia, which can be combatted by GRO therapy. Thus, GRO is broadly useful for treatment of hematopoietic cell deficiencies, whether congenital or therapy-induced.

GRO therapy can be accomplished by treating the patient systemically with an intravenous bolus or infusion of GRO (e.g. 1-100 μg/kg body weight per day) in a pharmaceutically effective carrier, or by any other effective means (such as an oral dose of a GRO analog that retains its potency when so administered, or by localizing the GRO injection directly to the *in vivo* site of the bone marrow to be treated). Alternatively, a preparation of the patient's bone marrow (or the bone marrow of a donor) can be treated *ex vivo* with GRO, cultured for

a period to permit generation of hematopoietic cells, and then implanted in the patient.

Other embodiments are within the following claims.

What is claimed is:

Claims

1. A method for promoting maturation of a hematopoietic precursor cell of an animal, said method comprising

providing GRO, and

contacting said cell with a maturation-promoting amount of said GRO.

2. The method of claim 1, wherein said cell is removed from said animal prior to contacting said cell with said GRO.

3. The method of claim 2, wherein said cell is reinserted into said animal after contacting said cell with said GRO.

4. The method of claim 2, wherein a descendent of said cell is inserted into said animal after contacting said cell with said GRO.

5. The method of claim 2, wherein said cell is inserted into a second animal after contacting said cell with said GRO.

6. The method of claim 2, wherein a descendent of said cell is inserted into a second animal after contacting said cell with said GRO.

7. The method of claim 5 or claim 6, wherein said second animal is a human.

8. A method for promoting the maturation of a hematopoietic precursor cell within an animal, said method comprising the steps of:

providing GRO, and

treating said animal with a maturation-promoting amount of said GRO.

9. The method of claim 1, 2, 3, 4, 5, 6, or 8 wherein said animal is a human.

10. The method of claim 1 or claim 8, wherein said GRO is a mature human GRO.

11. The method of claim 1 or claim 8, wherein said GRO is a recombinant GRO.

12. The method of claim 1 or claim 8, wherein said GRO is an analog or fragment of a naturally-occurring GRO.

13. The method of claim 1 or claim 8, wherein said cell is selected from a CFU-GEMM cell and a CFU-GM cell.

14. A method for promoting the maturation of a hematopoietic precursor cell within an animal, said method comprising the steps of

providing a nucleic acid molecule encoding GRO, and

introducing said nucleic acid molecule into a cell of said animal to form a transgenic cell which (a) expresses said GRO from said nucleic acid and (b) excretes said GRO in an amount sufficient to promote maturation of said hematopoietic precursor cell.

15. A method for promoting the maturation of a hematopoietic precursor cell within an animal, said method comprising the steps of

providing a second cell capable of excreting GRO, and

introducing said second cell into said animal to produce in said animal an amount of GRO sufficient to promote said maturation of said hematopoietic precursor cell.

FIG. 1

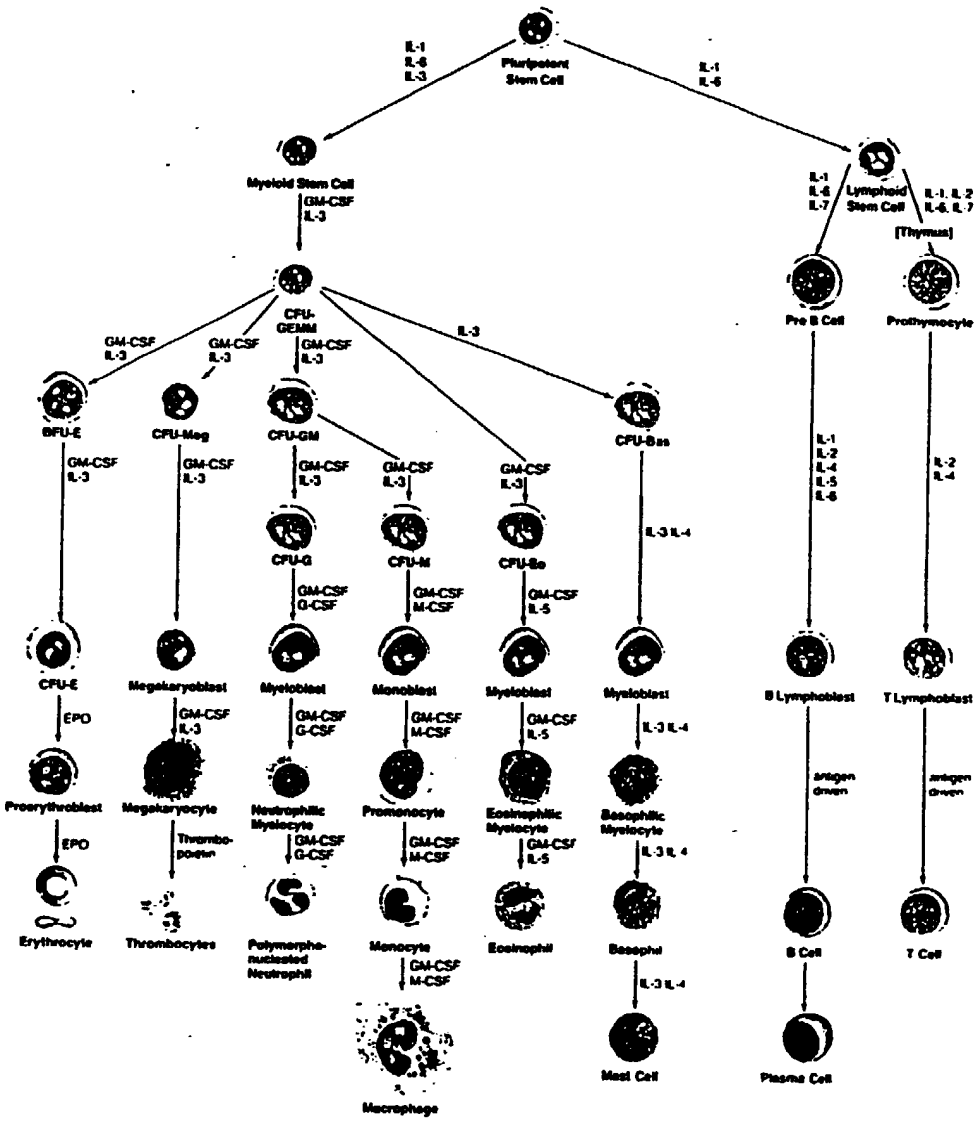

IL-1
IL-6
IL-3
Pluripotent Stem Cell
IL-1
IL-6
Myeloid Stem Cell
GM-CSF
IL-3
IL-1
IL-6
IL-7
Lymphoid Stem Cell
IL-1, IL-2
IL-6, IL-7
[Thymus]
CFU-GEMM
GM-CSF
IL-3
IL-3
Pre B Cell
Prothymocyte
BFU-E
CFU-Meg
CFU-GM
CFU-Bas
CFU-G
CFU-M
CFU-Eo
IL-1
IL-2
IL-4
IL-5
IL-6
IL-2
IL-4
IL-3 IL-4
GM-CSF
G-CSF
GM-CSF
M-CSF
GM-CSF
IL-5
CFU-E
Megakaryoblast
Myeloblast
Monoblast
Myeloblast
Myeloblast
B Lymphoblast
T Lymphoblast
EPO
Proerythroblast
Megakaryocyte
Neutrophilic Myelocyte
Promonocyte
Eosinophilic Myelocyte
Basophilic Myelocyte
antigen driven
antigen driven
Thrombo-poietin
Erythrocyte
Thrombocytes
Polymorpho-nucleated Neutrophil
Monocyte
Eosinophil
Basophil
B Cell
T Cell
Mast Cell
Plasma Cell
Macrophage

FIG. 2 (A)

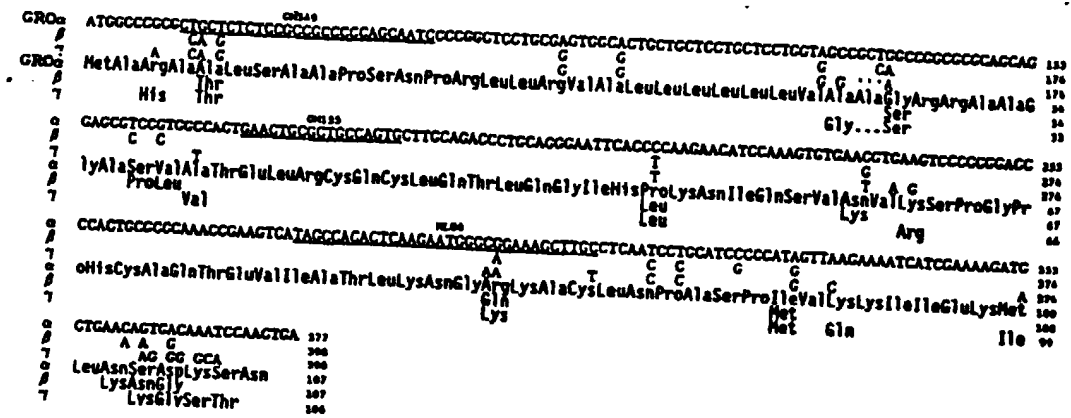


FIG. 2 (B)

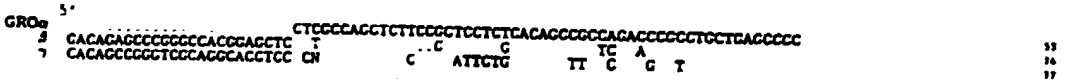


FIG. 2 (C)

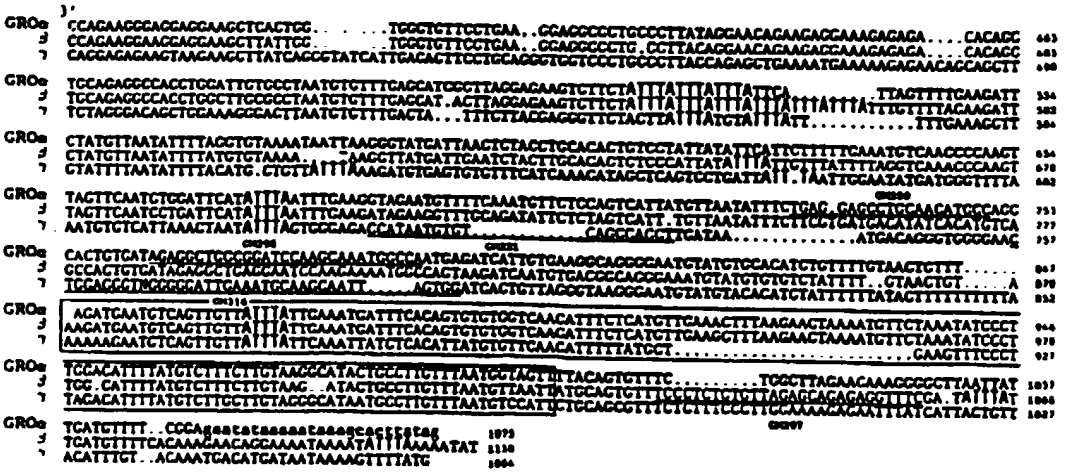


FIG. 3 (A)

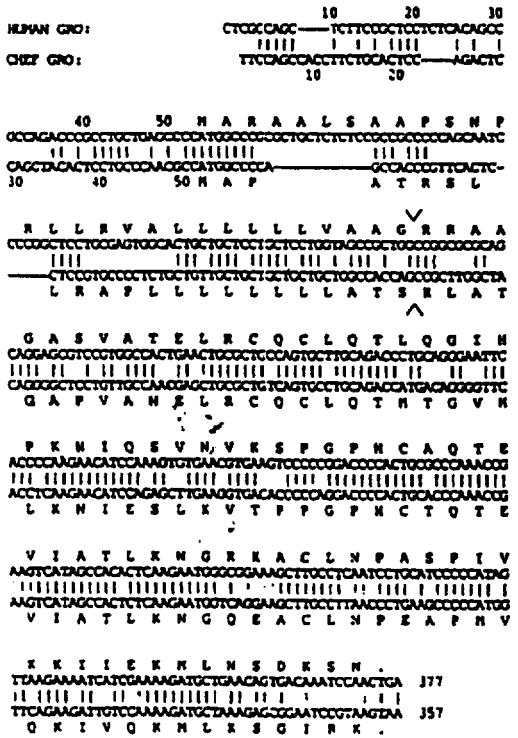


FIG. 3 (B)

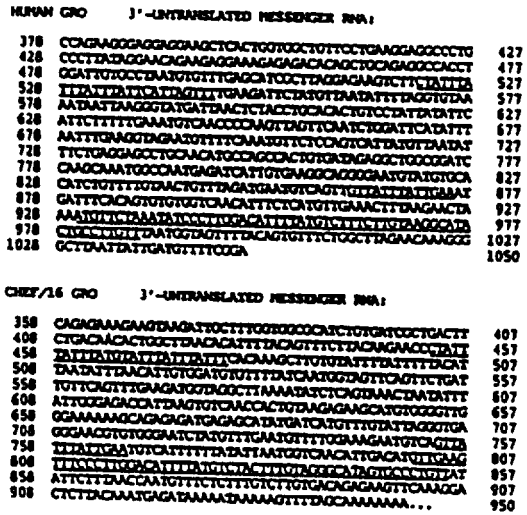
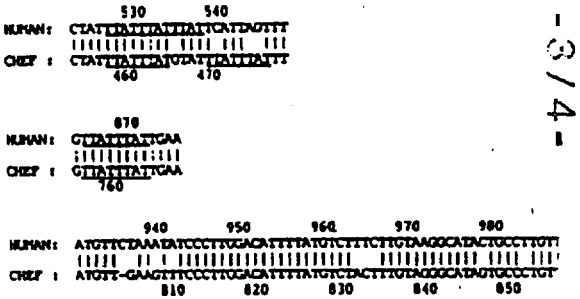
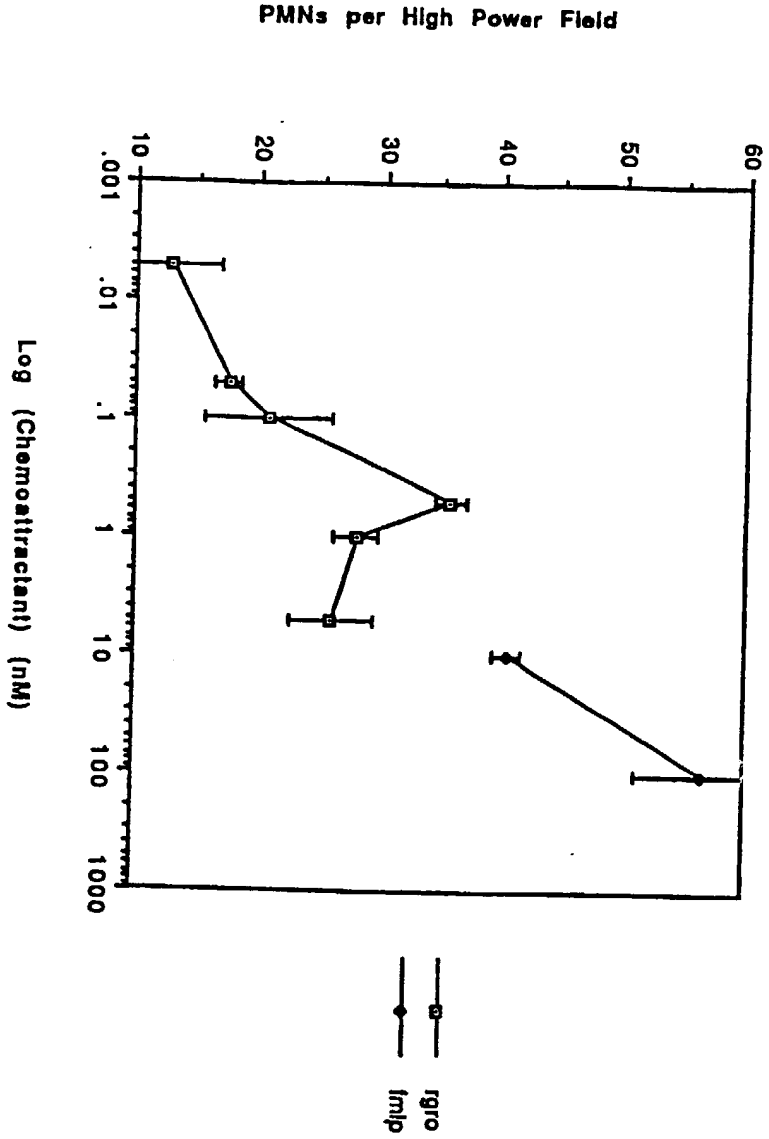


FIG. 3(C)





CHEMOTACTIC ACTIVITY OF rgro

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04885

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00, 5/08; C07K 13/00; A61K 37/36, 31/505, 35/14

U.S.Cl 435/240.1, 240.3, 424/85.1, 530 351

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US. Cl	435/240.1, 240. 3, 424/85.1, 534; 530/351

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 4,877,729 (Clark et al) 31 October 1989.	1-15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 September 1991	28 OCT 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	George C. Elliott George C. Elliott

Form PCT/ISA/210 (second sheet) (Rev.11-87)